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Original Article

Protective role of mineralocorticoid receptor signaling in urothelial tumorigenesis

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Abstract: The expression status of mineralocorticoid receptor (MR) and its biological significance in human urothelial carcinoma remain unknown. The present study aimed to determine the functional role of MR in the development of urothelial cancer. In human normal urothelial SVHUC cells with exposure to a chemical carcinogen 3-methylcholanthrene (MCA), we assessed the effects of a natural MR ligand, aldosterone, and 3 MR antagonists, including spironolactone, eplerenone, and esaxerenone, as well as knockdown of MR via shRNA virus infection, on their neoplastic/malignant transformation. The *in vitro* system with carcinogen challenge showed that aldosterone and anti-mineralocorticoids significantly prevented and promoted, respectively, the neoplastic transformation of SVHUC cells. Similarly, MR knockdown in SVHUC cells considerably induced MCA-mediated neoplastic transformation, compared with a control subline. In addition, MR knockdown or antagonist treatment resulted in increases in the expression of β-catenin, c-Fos, and N-cadherin, and a decrease in that of E-cadherin. Meanwhile, spironolactone, which is known to possess anti-androgenic activity, rather suppressed the neoplastic transformation of a SVHUC subline stably expressing wild-type androgen receptor, indicating its dominant effect via the androgen receptor pathway. Immunohistochemistry in surgical specimens detected MR signals in 77 (98.7%; 23.1% weak/1+, 42.3% moderate/2+, and 33.3% strong/3+) of 78 non-invasive bladder tumors, which was significantly (P<0.001) lower than in adjacent non-neoplastic urothelial tissues (100%; 20.5% 2+ and 79.5% 3+). Moreover, the risks for disease recurrence after transurethral surgery were marginally lower in female patients with MR-high (2+/3+) tumor (P=0.068) and significantly lower in all patients with MR-high/glucocorticoid receptor-high tumor (P=0.025), compared with respective controls. These findings suggest that MR signaling functions as a suppressor for urothelial tumorigenesis.

Keywords: Aldosterone, anti-mineralocorticoid, bladder cancer, eplerenone, esaxerenone, mineralocorticoid receptor, neoplastic transformation, spironolactone, urothelial carcinoma

Introduction

Urinary bladder tumor, mostly urothelial carcinoma, remains one of the most commonly diagnosed malignancies, especially in men, in various countries [1, 2]. Furthermore, the numbers of new bladder cancer cases and cancer related deaths throughout the world have even risen from 429,800 and 165,100 estimated in 2012 [1] to 573,278 and 212,536 reported in 2020 [2], respectively. Patients with bladder cancer often present with non-invasive disease, which can typically be managed with relatively conservative approaches including transurethral surgery. However, a considerable number of these patients following currently available intravesical pharmacotherapy have recurrence(s) occasionally with invasive tumor. Further identification of key molecules or signaling pathways responsible for the development of urothelial carcinoma is thus anticipated to ultimately provide targeted therapy options that more effectively prevent the occurrence and/or recurrence of bladder tumor.

In addition to various known exogenous risk factors for bladder cancer, some intrinsic factors have been implicated in the initiation and

progression of urothelial cancer. Specifically, a steroid hormone receptor, androgen receptor (AR), has been shown to be a crucial transcriptional regulator in not only prostate cancer but also others not yet being considered as endocrine-related neoplasms, such as bladder cancer [3, 4]. It has also been documented that estrogen receptors (ERs), including ERα and ERβ, contribute to modulating urothelial cancer outgrowth [5]. Moreover, our data suggested that another steroid hormone receptor, glucocorticoid receptor (GR), functions as a suppressor for urothelial tumorigenesis [6, 7]. Remarkably, recent studies have indicated the prognostic significance of steroid hormone receptor signals and related pathways in patients with urothelial cancer [8].

Mineralocorticoid receptor (MR) also belongs to the nuclear receptor superfamily as a ligand-inducible transcription factor. Physiologically, aldosterone, the major mineralocorticoid secreted by the adrenal cortex, is essential for sodium retention in the kidney and several other organs [9]. Clinically, anti-mineralocorticoids, such as spironolactone, eplerenone, and a new non-steroidal selective MR antagonist esaxerenone, have been used as diuretic agents for the treatment of, for example, hypertension, heart failure, chronic kidney disease, and primary aldosteronism [10]. Meanwhile, some of glucocorticoids (primarily as agonists), as well as other steroids, including progesterone (as an antagonist), have binding affinity for MR [10, 11]. Of note, prednisone, a glucocorticoid medication having mineralocorticoid potency, has been found to prevent the neoplastic transformation of urothelial cells, whereas other potent synthetic glucocorticoids with no or little agonist activity at MR, including dexamethasone and betamethasone, have shown no such inhibitory effects [6]. Furthermore, spironolactone possesses anti-androgenic and progestational activities, while other anti-mineralocorticoids, including eplerenone and esaxerenone, specifically bind to MR [10].

A case-control study involving a total of 64,334 patients demonstrated that spironolactone treatment significantly reduced the risk of developing bladder cancer in females [12], although its suppressive effect could be mediated via the AR pathway rather than the MR pathway. To the best of our knowledge, however, no preclinical studies have indicated the implication of MR signaling in the development and growth of urothelial cancer. In the present study, we investigated the impact of MR activation/inactivation on urothelial tumorigenesis, primarily using *in vitro* models.

Materials and methods

Cell culture and chemicals

An immortalized human normal urothelial line SVHUC and a human embryonic kidney cell line 293T were originally obtained from the American Type Culture Collection. A histiocytic lymphoma line U937 was a kind gift from Dr. Michael W. Becker (University of Rochester Medical Center, Rochester, NY, USA). SVHUC sublines stably expressing human full-length wild-type AR (*i.e.* SVHUC-AR) or vector only (*i.e.* SVHUC-control), as well as non-silencing control short hairpin RNA (shRNA) (*i.e.* SVHUC-control-shRNA), were established in our previous studies [13, 14]. Similarly, MR-shRNA lentiviral particles (sc-38836-V, Santa Cruz Biotechnology) were stably expressed in SVHUC cells. Multiple frozen aliquots were made upon the acquisition or establishment, and all experiments were performed with cells undergoing fewer than 20 passages. SVHUC and its sublines, 293T, and U937 were maintained in Ham's F-12K (Mediatech), Dulbecco's modified Eagle's medium (Gibco), and RPMI-1640 (Mediatech), respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 units/mL) at 37°C in a humidified atmosphere of 5% CO_2. We purchased aldosterone, spironolactone, and eplerenone from Cayman Chemical, and esaxerenone from ChemScene.

In vitro transformation

An *in vitro* neoplastic/malignant transformation system, using SVHUC-derived cells with exposure to 3-methylcholanthrene (MCA), was employed, as previously established [15], with minor modifications. Briefly, cells (2×10^6/10-cm culture dish incubated for 48 hours) were cultured in serum-free medium containing 5 μg/mL MCA (Sigma). After 24 hours of MCA exposure, 1% FBS was added to the medium. After additional 24 hours, the cells were cultured in medium containing 5% FBS without MCA until near confluence. Subcultured cells (1/3 split) were incubated in the presence of

MCA for two additional 48-hour exposure periods, using the above protocol. These MCA-exposed cells were then subcultured for 6 weeks in the presence or absence of an MR agonist or antagonist and thereafter utilized for further assays.

Cell proliferation

The methylthiazolyldisphenyl-tetrazolium bromide (MTT) assay was used to assess the cell viability. Cells (3-5 × 10^3/well) seeded in 96-well tissue culture plates were incubated for 96 hours, and at the end of the culture 10 µL MTT stock solution (5 mg/mL; Sigma) was added to each well for 3 hours. The medium was replaced with 100 µL dimethyl sulfoxide and incubated for 5 minutes at room temperature. The absorbance was then measured at a wavelength of 570 nm with background subtraction at 630 nm.

Colony formation

The colony formation assay was used to assess the clonogenic potential. Cells (1 × 10^3/well) seeded in 6-well tissue culture plates were allowed to grow until colonies in the control well were certainly detectable. The cells/colonies were then fixed with methanol and stained with 0.1% crystal violet. The number of colonies in photographed pictures was quantitated, using ImageJ software (National Institutes of Health).

Cell migration

A scratch wound-healing assay was used to assess the ability of cell migration. Cells at a density of 90-100% confluence in 6-well tissue culture plates were scratched manually with a sterile 200 µL plastic pipette tip. The cells were incubated in serum-free medium for 24 hours, fixed with methanol, and stained with 0.1% crystal violet. The width of the wound area was then quantitated, using the ImageJ.

Western blotting

Cell pellets from 6-well tissue culture plates were lysed in RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor cocktail; Thermo Scientific). Equal amounts (30 µg) of proteins extracted from the cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). After transferring onto a polyvinylidene difluoride membrane electronically, the membrane was blocked with 3% bovine serum albumin (for MR) or 3% milk (for GAPDH) and incubated with a primary antibody against MR (clone ab2774, diluted 1:250, Abcam) or GAPDH (clone 6C5, diluted 1:2000, Santa Cruz Biotechnology) at 4°C overnight, followed by 1-hour incubation with an HRP-conjugated secondary antibody (Cell Signaling Technology) at room temperature. Chemiluminescent signals were then detected using the ChemiDOC™ MP (Bio-Rad) imaging system.

We also used Simple Western™ system [16, 17]. Proteins (4 µg) were separated by size (12-230 kDa) and the signals were visualized on a Wes system with the Compass software (version 6.0.0) (ProteinSimple). The primary antibody against MR (clone ab2774, diluted 1:50, Abcam), β-catenin (clone E-5, diluted 1:50, Santa Cruz Biotechnology), c-Fos (clone sc-52, diluted 1:50, Santa Cruz Biotechnology), E-cadherin (clone sc-8426, diluted 1:50, Santa Cruz Biotechnology), N-cadherin (clone ab12-221, diluted 1:250, Abcam), or GAPDH (clone G-9, diluted 1:250, Santa Cruz Biotechnology) was used.

Immunohistochemistry

Bladder tissue microarray (TMA) consisting of non-invasive papillary urothelial neoplasms, along with adjacent normal-appearing urothelial tissues, were constructed, upon approval by the institutional review boards, as previously described [18, 19]. None of the patients had received therapy with radiation or anti-cancer drugs prior to the collection of the tissues included in the TMA. Immunohistochemical staining was performed on the sections (5 µm thick), using a primary antibody against MR (clone ab2774, dilution 1:100, Abcam), as previously described [13, 18-20]. Positive controls for the stain (*e.g.* kidney, colon) and negative tissue elements were both evaluated and were adequate for the interpretation in the bladder TMA. All stains were manually quantified by a single pathologist (H.M.) who was blinded to sample identify. The immunoreactive scores (range: 0-12) calculated by multiplying the percentage of immunoreactive cells (0%=0; 1-10%=1; 11-50%=2; 51-80%=3; 81-100%=4) by staining intensity (negative =0; weak =1; moderate =2; strong =3) were considered negative (0; 0-1), weakly positive (1+; 2-4), moder-

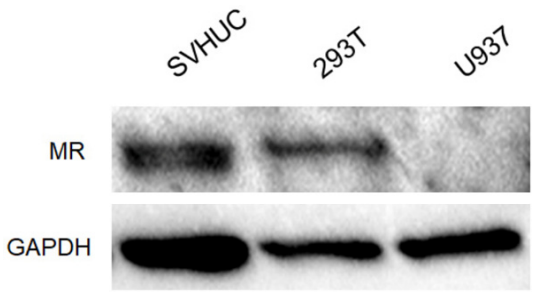

Figure 1. MR expression in a non-neoplastic urothelial cell line. Western blotting of MR in SVHUC, 293T (as a positive control), and U937 (as a negative control). GAPDH served as a loading control.

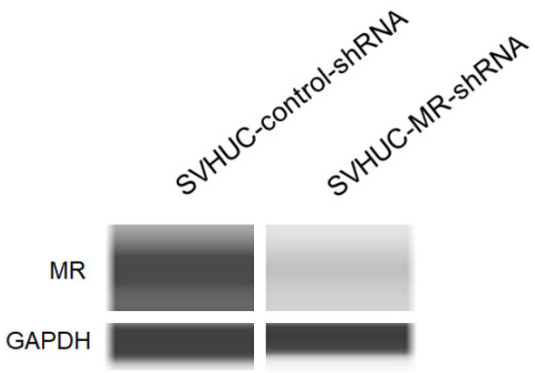

Figure 2. MR knockdown in SVHUC cells. Western blotting (Simple Western™ system) of MR in SVHUC-control-shRNA vs. SVHUC-MR-shRNA. GAPDH served as a loading control.

ately positive (2+; 6-8), and strongly positive (3+; 9-12).

Statistical analysis

Student's *t*-test was used to compare numerical data. Fisher's exact test or chi-square test was used to evaluate the associations between categorized variables. Survival rates in patients were calculated by the Kaplan-Meier method and comparison was made by Gehan-Breslow-Wilcoxon test. *P* values of less than 0.05 were considered statistically significant.

Results

Expression of MR in urothelial cells and tissues

We first examined the status of MR expression in non-neoplastic urothelial cells, using western blotting, along with adequate positive and negative controls [21]. An immortalized human normal urothelial line SVHUC, which had been shown to be AR-negative [13], was thus found to express the MR (**Figure 1**). We then silenced MR in SVHUC cells via its shRNA. We confirmed considerable reduction in MR expression in a knockdown subline (**Figure 2**).

We next stained immunohistochemically for MR in a set of bladder TMA consisting of 78 non-invasive papillary urothelial neoplasm specimens and corresponding 39 non-neoplastic bladder tissues (**Figure 3A**, **3B**). Overall, MR was positive in 98.7% (23.1% 1+, 42.3% 2+, and 33.3% 3+) of tumors, which was significantly weaker than in benign urothelial tissues [100% (20.5% 2+ and 79.5% 3+)] (**Table 1**). However, there were no significant differences in the status of MR expression between tumors from male vs. female patients or low grade (*i.e.* papillary urothelial neoplasm of low malignant potential + low-grade urothelial carcinoma) vs. high-grade tumors. Kaplan-Meier analysis revealed that the risk of recurrence after transurethral resection of the tumors was lower in patients with MR-high (2+/3+) tumor than in those with MR-low (0/1+) tumor, although the difference was not statistically significant (*P*=0.129; **Figure 3C**). The difference was slightly more distinct in female patients (*P*=0.068; **Figure 3D**), but not in male patients (*P*=0.811). Meanwhile, in our previous study [16], GR had been stained in the bladder tumors included in the same set of TMA (*i.e.* 3.8% 0, 23.1% 1+, 43.6% 2+, 29.5% 3+), while a correlation of MR vs. GR expression was not high (correlation coefficient 0.176, *P*=0.081). When considering the expression status of both MR and GR, patients with MR-high/GR-high tumor had a significantly lower risk of recurrence (*P*=0.025; **Figure 3E**).

Impact of MR activation/inactivation on the neoplastic transformation of urothelial cells

We used an established *in vitro* model where non-neoplastic SVHUC cells could undergo the neoplastic/malignant transformation induced by a chemical carcinogen MCA during the course of 6-week culture [6, 7, 15, 22]. First, SVHUC cells exposed to MCA for 48 hours three times were subcultured in the presence or absence of MR ligands in medium containing normal FBS for 6 weeks during the process of transformation. Oncogenic activity in the trans-

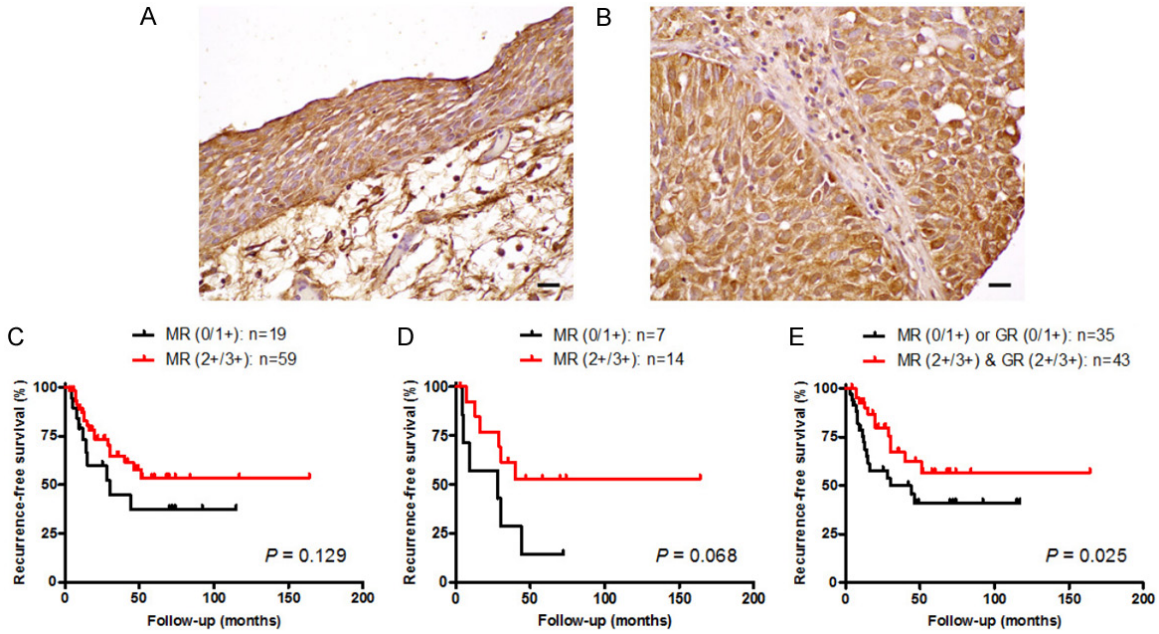

Figure 3. Immunohistochemistry of MR in bladder tumors. Representative images of MR expression in normal urothelial tissue (A) and urothelial tumor (B). Original magnification: 400 × (scale bar 20 μm). Kaplan-Meier curves for recurrence-free survival in 78 patients (C) or 21 female patients (D) with non-invasive papillary urothelial neoplasm, according to the status of MR expression, as well as in 78 patients with non-invasive papillary urothelial neoplasm, according to the status of MR and GR expression (E).

Table 1. Correlations of MR expression with clinicopathologic profile of the patients with non-invasive disease

		MR expression				*P* value	
	n	Negative	Positive			0/1+ vs. 2+/3+	0/1+/2+ vs. 3+
		0	1+	2+	3+		
Tissue						<0.001	<0.001
Non-neoplastic epithelium	39	0 (0%)	0 (0%)	8 (20.5%)	31 (79.5%)		
Urothelial neoplasm	78	1 (1.3%)	18 (23.1%)	33 (42.3%)	26 (33.3%)		
Sex						0.372	0.787
Male	57	1 (1.8%)	11 (19.3%)	25 (43.9%)	20 (35.1%)		
Female	21	0 (0%)	7 (33.3%)	8 (38.1%)	6 (28.6%)		
Tumor grade						0.266	0.803
PUNLMP + LG	52	1 (1.9%)	14 (26.9%)	19 (36.5%)	18 (34.6%)		
HG	26	0 (0%)	4 (15.4%)	14 (53.8%)	8 (30.8%)		

PUNLMP, Papillary Urothelial Neoplasm of Low Malignant Potential; LG, Low-Grade papillary urothelial carcinoma; HG, High-Grade papillary urothelial carcinoma.

formed cells was then monitored by subsequent assays for cell viability (via MTT assay; **Figure 4A**), colony formation (via clonogenic assay; **Figure 4B**), and cell migration (via wound-healing assay; **Figure 4C**) with no further drug treatment that could directly affect their results. We thus compared the degree of neoplastic transformation in urothelial cells with carcinogen challenge but did not intend to simply assess the effects of MR agonist/antagonists on the growth of SVHUC-derived cells. Aldosterone treatment in MCA-SVHUC cells significantly inhibited their neoplastic transformation in all assays, while three anti-mineralocorticoids, spironolactone, eplerenone, and esaxerenone, similarly induced it.

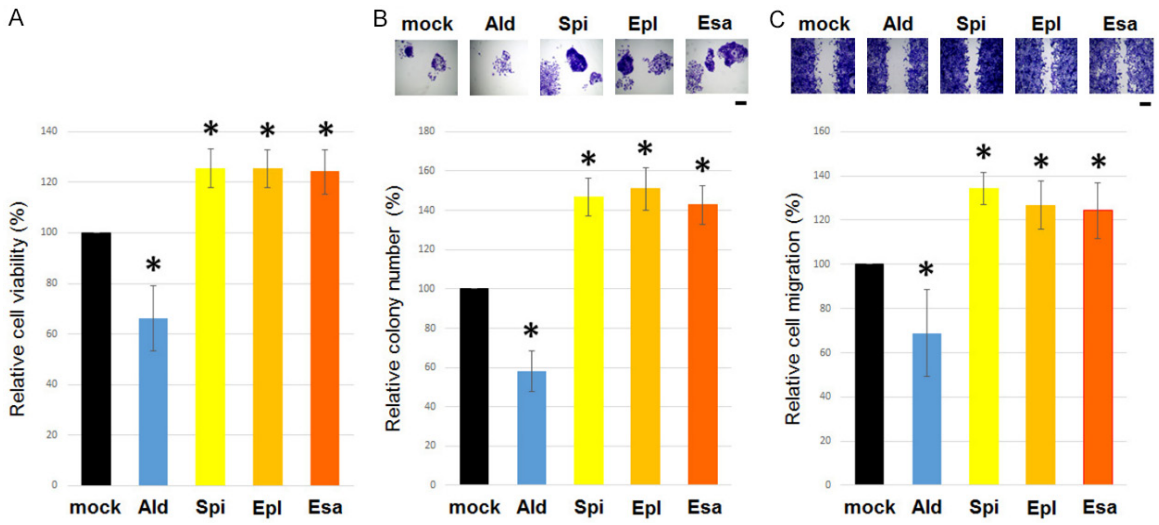

Figure 4. Effects of an MR agonist and MR antagonists on the neoplastic transformation of urothelial cells. SVHUC exposed to MCA and subsequently cultured with ethanol (mock), aldosterone (Ald; 10 nM), spironolactone (Spi; 1 μM), eplerenone (Epl; 1 μM), or esaxerenone (Esa; 10 nM), as indicated, for 6 weeks were seeded for MTT assay (A, additional 96-hour culture), clonogenic assay (B, additional 2-week culture), or wound-healing assay (C, additional 24-hour culture) without further drug treatment. Cell viability, colony number (≥20 cells), or width of the wound area presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments. The scale bars under the images indicate 200 μm. *P<0.05 (vs. mock-treated cells).

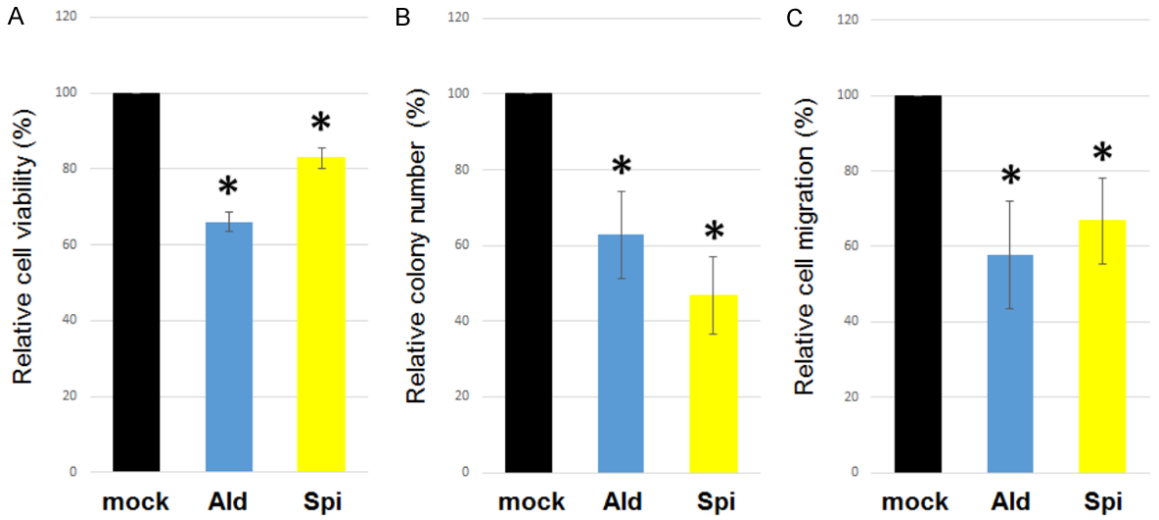

Figure 5. Effects of spironolactone on the neoplastic transformation of AR-positive urothelial cells. SVHUC-AR exposed to MCA and subsequently cultured with ethanol (mock), aldosterone (Ald; 10 nM), or spironolactone (Spi; 1 μM), as indicated, for 6 weeks were seeded for MTT assay (A, additional 96-hour culture), clonogenic assay (B, additional 2-week culture), or wound-healing assay (C, additional 24-hour culture) without further drug treatment. Cell viability, colony number (≥20 cells), or width of the wound area presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments. *P<0.05 (vs. mock-treated cells).

AR signaling has been implicated in the development of urothelial cancer [4]. In particular, anti-androgenic compounds have been shown to strongly prevent urothelial tumorigenesis [3, 7, 22]. Because spironolactone was known to possess anti-androgenic activity [10], its efficacy in AR-positive urothelial cells were also assessed (**Figure 5**). In MCA-SVHUC-AR cells, aldosterone with no known AR activity still inhibited their neoplastic transformation. Similarly, treatment with spironolactone resulted in rather significant reduction in the neo-

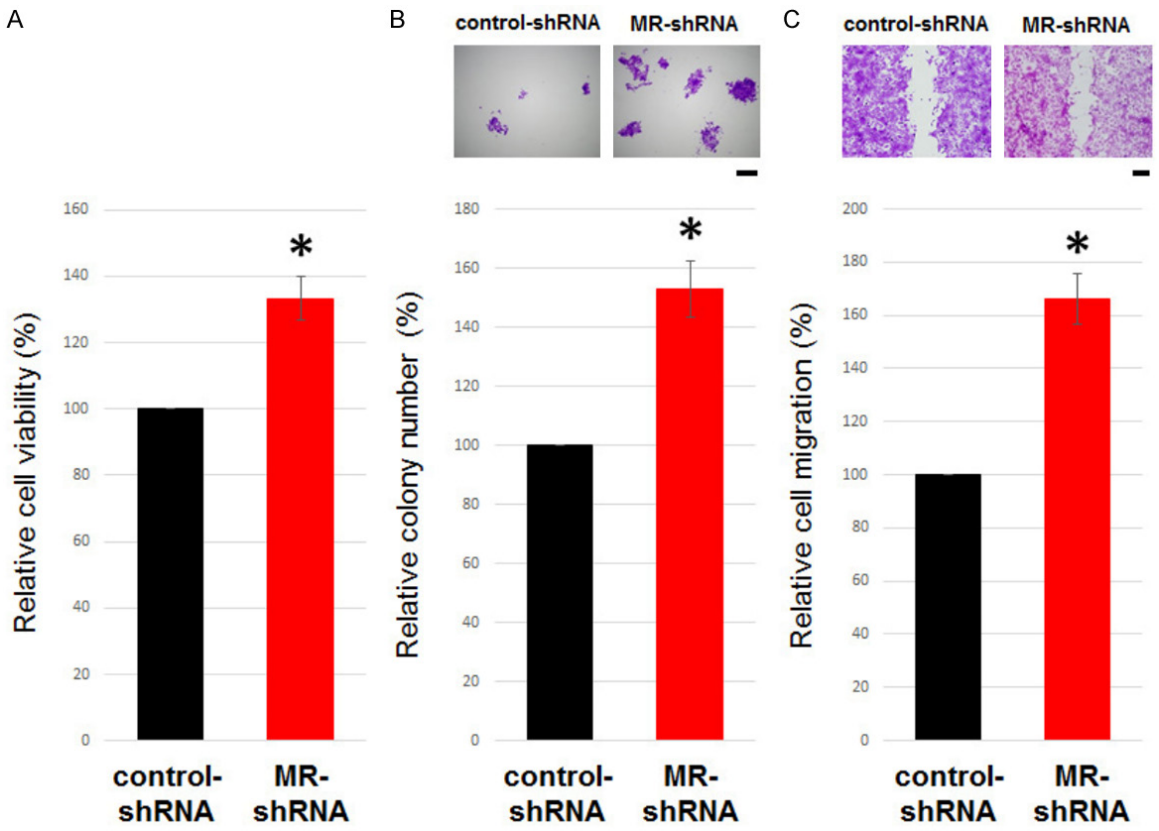

Figure 6. Effects of MR knockdown on the neoplastic transformation of urothelial cells. SVHUC-control/MR-shRNA sublines exposed to MCA and subsequently cultured for 6 weeks were seeded for MTT assay (A, additional 96-hour culture), clonogenic assay (B, additional 2-week culture), or wound-healing assay (C, additional 24-hour culture) without drug treatment. Cell viability, colony number (≥20 cells), or width of the wound area presented relative to that of control-shRNA subline represents the mean (±SD) from three independent experiments. The scale bars under the images indicate 100 μm (B) and 200 μm (C), respectively. *P<0.05 (vs. control-shRNA subline).

plastic transformation of MR-positive/AR-positive cells, implying the inhibitory effect of spironolactone predominantly via the AR pathway.

To further investigate the functional role of MR in the neoplastic transformation of urothelial cells, we compared MCA-induced oncogenic activity of control (SVHUC-control-shRNA) vs. knockdown (SVHUC-MR-shRNA) sublines. As expected, MR knockdown resulted in significant increases in cell viability (**Figure 6A**), colony formation (**Figure 6B**), and cell migration (**Figure 6C**), compared with the control. These findings suggest the protective function of MR signaling in urothelial tumorigenesis.

Expression of MR vs. oncogenic proteins in urothelial cells

We finally investigated the impact of MR on the expression of several molecules known to involve urothelial tumorigenesis, including β-catenin [23], c-Fos [14], E-cadherin [24], and N-cadherin [25]. Western blotting in SVHUC-control-shRNA vs. SVHUC-MR-shRNA showed that the expression levels of MR and β-catenin/c-Fos/N-cadherin were inversely correlated, while E-cadherin expression was down-regulated in MR knockdown cells (**Figure 7A**). In addition, treatment with an antagonist resulted in similar changes in the expression of these four proteins (**Figure 7B**).

Discussion

Previous studies in cell line models have suggested that the activity of MR, primarily as a suppressor, is associated with the progression of several types of malignancies, such as breast cancer [26, 27], glioblastoma [28], liver cancer [29], and prostate cancer [30]. By contrast, no preclinical evidence has supported the involvement of MR signaling in tumorigenesis which is generally considered to be a pro-

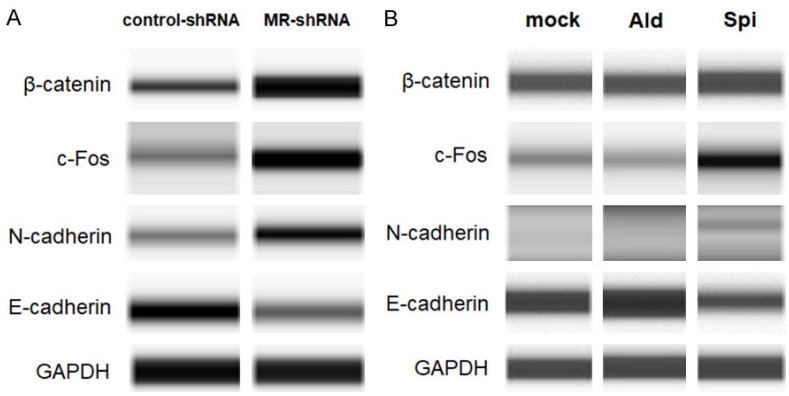

Figure 7. Effects of MR knockdown or ligand treatment on the expression of oncogenic molecules in urothelial cells. Western blotting (Simple Western™ system) of β-catenin, c-Fos, N-cadherin, and E-cadherin in SVHUC-control-shRNA vs. SVHUC-MR-shRNA (A) or SVHUC cells cultured with ethanol (mock) vs. aldosterone (Ald; 100 nM) vs. spironolactone (Spi; 10 μM) for 24 hours (B). Representative images for GAPDH, which serves as a loading control, are shown.

cess distinct from tumor progression. Instead, retrospective studies have explored the risks of developing malignant neoplasms in those treated with spironolactone but have identified no considerable impact of spironolactone exposure on the incidence of various types of cancer examined [31]. Only an exception is prostate cancer whose incidence has been shown to be rather significantly reduced in men with spironolactone [12, 31-34], but not with other classes of anti-hypertensive agents [12, 33]. Considering not only the stimulatory effects of MR antagonists on the growth of a prostate cancer cell line [30], but also AR dependence in prostate carcinogenesis, spironolactone is likely to prevent its development as an anti-androgen, but not as an anti-mineralocorticoid. Additionally, as aforementioned, a case-control study demonstrated the lower incidence of bladder cancer in females with spironolactone (odds ratio 0.81, $P<0.001$) [12], while no significant difference in males (odds ratio 1.03, $P>0.05$) was unexplainable by its effect predominantly through the AR pathway. There are no epidemiological data on the cancer risk in those treated with other anti-mineralocorticoids. In the present study, we therefore investigated the functional role of MR in urothelial tumorigenesis.

Using a carcinogen-mediated *in vitro* system, we compared oncogenic activity (via cell viability, colony formation, and cell migration) and first found that an agonist (*i.e.* aldosterone) and antagonists (*i.e.* spironolactone, eplerenone, esaxerenone) for MR inhibited and promoted, respectively, the neoplastic transformation of urothelial cells. Specifically, three anti-mineralocorticoids at the doses close to their pharmacological concentrations showed similar induction. These findings suggested that MR could prevent urothelial carcinogenesis. This was further confirmed by the induction of the carcinogen-mediated neoplastic transformation by MR knockdown. We then examined the potential downstream targets that had been known to involve urothelial tumorigenesis [14, 23-25]. We found that MR expression/activity in normal urothelial cells was positively associated with that of E-cadherin, which is often lost during malignant transformation [24], and that MR knockdown or antagonist treatment enhanced the expression of oncogenic molecules, including β-catenin, c-Fos, and N-cadherin. Nonetheless, further studies, particularly in *in vivo* models, are required to validate our results. The molecular mechanisms responsible for MR-mediated suppression of urothelial tumorigenesis should also be further elucidated. In particular, MR has been documented to functionally interact with other steroid hormone receptors, such as GR and AR [10, 11, 35, 36], each of which is known to, by itself, modulate the development of bladder cancer [3, 4, 6, 7].

Our *in vitro* data suggesting the protective role of MR signaling in urothelial tumorigenesis were supported by immunohistochemical findings in surgical specimens. Indeed, down-regulation of the expression of MR mRNA or protein has been demonstrated in several cancer types, such as colorectal carcinoma [37], hepatocellular carcinoma [29], and renal cell carcinoma [38]. In addition, pan-cancer assessment of The Cancer Genome Atlas data revealed that *MR* gene expression was lowered in bladder cancer [39]. In line with these observations, we here found that MR expression was considerably down-regulated in non-invasive bladder tumors, compared with normal-appearing urothelial tissues. Moreover, the risk of tumor

recurrence after transurethral resection, which might represent tumorigenesis, was marginally, yet not statistically significantly, lower in those with MR-high tumor, particularly in female patients. More strikingly, tumors showing both high MR and high GR were found to be strongly associated with a reduced risk of disease recurrence. The clinical significance of combined MR/GR expression might be particularly interesting, because the heterodimer of MR and GR could regulate transcription of the molecules, such as FKBP5 and PER1, that are distinct from those mediated by MR homodimers [11, 35, 36].

Again, spironolactone has been known to function as an antagonist for not only MR but also AR [10]. It is therefore more likely that the preventive role of spironolactone in the development of prostate cancer [12, 31-34] is mediated primarily via the AR pathway. Indeed, significant decreases in the levels of serum prostate-specific antigen, a reliable prostate cancer marker and a downstream target of AR signals, were observed in men treated with spironolactone, which was reversed by its discontinuation [32]. Then, if MR certainly acts as a tumor suppressor, it is possible that the stimulatory and inhibitory effects of spironolactone are dependent on the functional activity of MR versus AR in those where both are expressed. In the present study, we demonstrated that spironolactone significantly promoted and prevented the neoplastic transformation of MR-positive/AR-negative and MR-positive/AR-positive urothelial cells, respectively. This suggests that AR plays a more dominant role in urothelial tumorigenesis, compared with MR. However, because a subline stably overexpressing AR was used, another non-neoplastic urothelial line with endogenous AR/MR expression, which does not appear to be commercially available, might need to be tested to determine the true predominance of MR vs. AR. It is worthy to mention that MR appears to be expressed, although down-regulated, in the majority of bladder tumors as we here demonstrated, whereas immunohistochemical studies have shown loss of AR expression in 46.6-87.5% of cases [4, 8, 16, 40].

In conclusion, the present results suggest that MR signaling functions as a suppressor for urothelial tumorigenesis, which may then offer a potential preventive strategy for the development of bladder cancer via activation of the MR pathway. On the other hand, treatment with anti-mineralocorticoids, especially eplerenone and esaxerenone, for, for example, hypertension and heart failure, might be harmful to those concurrently with urothelial cancer or otherwise high-risk patients. Further assessment of MR functions is required to determine its biological significance in urothelial tumorigenesis. Mechanistic details underlying the suppression of tumor development by MR signaling also need to be further characterized.

Disclosure of conflict of interest

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